THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 24, Issue of June 16, pp. 18586–18593, 2000
Printed in U.S.A.

TTRAP, a Novel Protein That Associates with CD40, Tumor Necrosis Factor (TNF) Receptor-75 and TNF Receptor-associated Factors (TRAFs), and That Inhibits Nuclear Factor-κB Activation*

Received for publication, January 20, 2000, and in revised form, April 7, 2000
Published, JBC Papers in Press, April 11, 2000, DOI 10.1074/jbc.M000531200

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CD40 belongs to the tumor necrosis factor (TNF) receptor family. CD40 signaling involves the recruitment of TNF receptor-associated factors (TRAFs) to its cytoplasmic domain. We have identified a novel intracellular CD40-binding protein termed TRAF and TNF receptor-associated protein (TTRAP) that also interacts with TNF-R75 and CD30. The region of the CD40 cytoplasmic domain that is required for TTRAP association overlaps with the TRAF6 recognition motif. Association of TTRAP with CD40 increases profoundly in response to treatment of cells with CD40L. Interestingly, TTRAP also associates with TRAFs, with the highest affinity for TRAF6. In transfected cells, TTRAP inhibits in a dose-dependent manner the transcriptional activation of a nuclear factor-κB (NF-κB)-dependent reporter mediated by CD40, TNF-R75 or Phorbol 12-myristate 13-acetate (PMA) and to a lesser extent by TRAF2, TRAF6, TNF-α, or interleukin-1β (IL-1β). TTRAP does not affect stimulation of NF-κB induced by overexpression of the NF-κB-inducing kinase (NIK), the IκB kinase α (IKKα), or the NF-κB subunit P65/RelA, suggesting it acts upstream of the latter proteins. Our results indicate that we have isolated a novel regulatory factor that is involved in signal transduction by distinct members of the TNF receptor family.

CD40 is a member of the tumor necrosis factor (TNF)[1] receptor family that plays a critical role in many immunological processes (1). The receptor is present on many cell types, and its function has been studied most extensively in B cells, dendritic cells, monocytes, and endothelial cells. Characterization of mice deficient for CD40 or its ligand CD40L (also named CD154) highlights the importance of CD40-mediated signaling in the thymus-dependent humoral immune response and in isotype switching (2–4). CD40-mediated signal transduction induces the transcription of a large number of genes implicated in host defense against pathogens. This is accomplished by the activation of multiple transcription factors, including NF-κB (5), c-Jun (6), and STAT3 (7). In the past 5 years we have come to understand in significant detail the cascade that leads from stimulation of TNF receptors to the activation of transcription factors. The signal transduction is triggered by binding of trimeric ligands of the TNF family to their cognate receptors, which induces oligomerization of the latter at the cell surface. This brings the intracellular domains of these receptors in close proximity whereby they serve as a high affinity binding platform for many cytoplasmic proteins involved in signal transduction.

Members of the TNF receptor family, such as CD30, CD40, TNF-R75, OX40, RANK, and 4–1BB, have been implicated primarily in gene activation rather than apoptosis and transmit their signal through the direct recruitment of TRAFs (8). TRAFs 1–6 display similar structural features, *i.e.* they have an N-terminal RING finger (which is absent in TRAF1), followed by 5–7 zinc fingers, and a C-terminal TRAF domain that mediates receptor binding. CD40 associates with TRAFs 2, 3, 5, and 6 (9–12). The importance of the latter for signaling by CD40 and other receptors has become clear from the characterization of TRAF6-deficient mice. Experiments performed with cells derived from these mice demonstrated that TRAF6 is

* This work was supported by a postdoctoral fellowship of the "Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie" (IWT) (to S. P.) and by funds from Innogenetics S. A., and the Flanders Interuniversitary Institute for Biotechnology, within the framework of a collaboration agreement (1998–2000). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ269473 and AJ251328.

‖ Supported by the "Interuniversitaire Attractiepolen" (IUAP 9005097N).

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¶¶ Supported by the Flanders Interuniversitary Institute for Biotechnology.

[1] The abbreviations used are: TNF, tumor necrosis factor; CCR4, carbon catabolite repressor protein; HA, hemagglutinin (tag); HUVEC, human umbilical vein endothelial cell; IKK, IκB-kinase; IL, interleukin; TRAF, TNF receptor-associated factor; I-TRAF, TRAF-interacting protein; JAK, Janus kinase; LMP1, latent membrane protein 1; MAPK, mitogen-activated kinase; MEKK1, MAPK/extracellular response kinase; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; PMA, phorbol 12-myristate 13-acetate; RANK, receptor activator of NF-κB; STAT, signal transducer and activator of transcription; TAK1, transforming growth factor-β-activated kinase; TANK, TRAF-associated NF-κB activator; TNF-R, TNF receptor; TRADD, TNF-R1-associated death domain protein; TRIP, TRAF-interacting protein; TTRAP, TRAF and TNF receptor-associated protein; EST, expressed sequence tag; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

crucial for CD40L, IL-1, and lipopolysaccharide-dependent activation of NF-κB (13). These results confirmed earlier observations that TRAF6 is involved in gene activation through members of the TNF receptor and IL-1 receptor families (11, 14–16). Recently, TRAF2 was also shown to be essential for CD40-mediated responses in mice (17). The molecular mechanisms by which TRAFs activate downstream effector proteins remain largely unknown. However, the current data suggest that this involves the interaction of TRAFs with different types of kinase. Some of these are involved in pathways leading to NF-κB activation, *e.g.* NIK (18), MEKK1 (19), and TAK1 (20). These kinases have the potential to activate the IκB kinases (IKKα and IKKβ) (21–23), which phosphorylate IκB. This phosphorylation triggers ubiquitination and subsequent degradation of IκB, resulting in the release of NF-κB subunits that translocate into the nucleus, where they act as transcription activators (reviewed in Ref. 24).

Signal transduction by members of the TNF receptor family also involves several regulatory factors. Most of these proteins have been identified as TRAF-binding proteins, *e.g.* A20 (25), I-TRAF/TANK (26, 27), and TRIP (28). Although their precise role in the signal transduction process remains elusive, overproduction of these factors either inhibits (in the case of A20, I-TRAF, TRIP) or synergistically activates (in the case of TANK) TRAF-mediated activation of NF-κB. As a result of a search for novel effector proteins involved in CD40 signaling, this study describes the identification of a novel regulatory protein that binds receptors and TRAFs and that inhibits activation of NF-κB.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Anti-FLAG M2 monoclonal antibody was purchased from Sigma and anti-CD40 polyclonal antibody C20, that was used for Western blot, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin (HA) tag monoclonal antibody was a gift from Innogenetics S. A. (Zwijnaarde, Belgium), and anti-hCD40 5D12 monoclonal antibody, used for immunoprecipitation, was from Tanox Pharma B. V. (Amsterdam, The Netherlands). The anti-hTNF-R75 mouse monoclonal antibody utr4 was a gift of M. Brockhaus and W. Lesslauer (Roche, Basel, Switzerland). The anti-hTNF-R75 polyclonal antibodies were from W. Buurman (University Maastricht, The Netherlands). The following expression vectors for production of human and murine TRAFs were a gift from D. Goeddel (Tularik Inc., South San Francisco, CA): FLAG-hTRAF2-pRK5, FLAG-ΔTRAF2-pRK5 (insert encodes amino acids 87–501 of mouse TRAF2 (10)), FLAG-hTRAF6-pRK5, and FLAG-Δ289TRAF6-pRK5 (insert encodes amino acids 289–511 of human TRAF6 (16)). FLAG-Δ317TRAF6-pcDNA3 was constructed by PCR amplification on FLAG-hTRAF6-pRK5, engineering an *Eco*RI site at the 5′-end of the partial cDNA and cloning the *Eco*RI-*Xho*I fragments into FLAG-pcDNA3. HA-hTRAF3-pcDNA3 was a gift from V. Dixit (University of Michigan, Ann Arbor, MI). HA-IKKα-pcDNA and Xpress-NIK-pcDNA3 were a gift from M. Karin (University of California San Diego, La Jolla, CA). FLAG-hTRAF5-pME was provided by J. Inoue (Tokyo University, Tokyo, Japan) and P65/RelA-pRc/cytomegalovirus by S. Plaisance (University of Gent, Gent, Belgium).

CD40 cDNA was amplified by PCR from a human umbilical vein endothelial cell (HUVEC) cDNA library and cloned into pcDNA3. The cDNAs encoding the cytoplasmic part of human CD40 (amino acids 216–277 (11)), human TNF-R75 (262–437 (29)), and human CD30 (408–595 (30)), were generated by PCR and inserted into the pEG202 vector (Gyuris *et al.* (32)) in-frame with the sequence encoding the LexA DNA-binding domain. The cDNA for the C-terminal cytoplasmic domain of LMP1 (amino acids 192–386 (31)) was obtained from M. Rowe (University of Wales, Cardiff, United Kingdom (UK)). Our CD40 deletion and point mutants were constructed by PCR, as described by Ishida and co-workers (11), and cloned into pEG202. Plasmid hTNF-R75-pcDNA6 was described previously (29), and human TRADD was cloned in frame with an N-terminal E tag, into pcDNA3.

4F2 and TRAF3 partial cDNAs that were picked in our two-hybrid screening were excised from pJG4–5, using *Eco*RI, and subcloned into the similarly digested vectors pEG202, FLAG-pcDNA3 and HA-pcDNA3. Full-length TTRAP cDNA was cloned in two ways. First, cloning into HA-pcDNA3 was done starting directly from the cDNA picked from the HUVEC library, via digestion with *Eco*RI and ligation into HA-pcDNA3. In doing so, 34 nucleotides from the library vector and 20 from the 5′-untranslated region of TTRAP cDNA are present between the sequence encoding the HA tag and the translation initiation codon of TTRAP. Second, for cloning of TTRAP cDNA in pJG4–5, a PCR-based approach was used. An *Eco*RI site was engineered directly adjacent to the 5′-end of the TTRAP cDNA by amplification of TTRAP cDNA using the primer combination 5′-GACGAATTCAGAGGCG-GCAGGAAGATGGAGTTGG and 5′-GCCTCACATCCTGAATGCAG-GA. The amplified fragment was then digested with *Eco*RI and *Bgl*II and ligated together with a *Bgl*II-*Nco*I TTRAP cDNA fragment into pJG4–5. FLAG-TTRAP-pcDNA3 and TTRAP-pCS2 were obtained by ligation of the *Eco*RI fragment from TTRAP-pJG4–5 into FLAG-pcDNA3 and pCS2, respectively.

Two-hybrid Screening—Two-hybrid screening in yeast was performed by the interaction trap cloning method, which is often referred to as the LexA two-hybrid system (32). The cytoplasmic part of human CD40 was cloned in-frame with the LexA DNA-binding domain (the bait plasmid). Screening was done using a HeLa cell cDNA library in pJG4–5 (the prey plasmid), which was obtained from R. Brent (Harvard Medical School, Boston, MA). EGY48 (MAT α, *his3, trp1, ura3–52, leu2::pLEU2-LexAop*) yeast cells were transformed with the prey plasmid, the bait plasmid, and the *lacZ* reporter plasmid pSH18–34 by the lithium acetate transformation method (33).

Yeast cells containing bait plasmid and *lacZ* reporter plasmid were transformed with 20 μg of library plasmid and plated on glucose medium lacking tryptophan, histidine and uracil, to select for the presence of all three plasmids. In total, approximately 2×10^6 colonies were obtained. These transformants were harvested and frozen at −80 °C in a glycerol solution (65% glycerol (v/v), 100 mM $MgSO_4$, 25 mM Tris/HCl pH 7.4). To screen for protein-protein interaction, 20×10^6 colony-forming units of an amplified stock of original transformants were tested for a positive interaction phenotype, as described (32). When using yeast two-hybrid as test for interaction, we performed mating assays (34). Bait and prey constructs were transformed in yeast strain EGY48 (mating type α) and EGY42 (mating type a), respectively.

Northern Blotting, in Situ Hybridization and Isolation of Full-length TTRAP cDNA—Northern analysis of human and murine mRNA blots (CLONTECH, Palo Alto, CA) was carried out with human 4F2 and the entire cDNA of mouse TTRAP (EST clone 876634) as probes, respectively. Blots were hybridized at 65 °C in QUICKHYB hybridization solution (Stratagene, La Jolla, CA). The 2-kb-long probe used for *in situ* hybridization was the same as used for Northern analysis of mouse *TTRAP*. *In vitro* transcription with T3 RNA Polymerase yielded [^{35}S]-uracil (NEN Life Science Products) labeled single-stranded riboprobe. *In situ* hybridization in sections of mouse embryos was done as described previously (35).

Full-length human TTRAP cDNA was obtained by screening a HUVEC cDNA plasmid library with human 4F2 as probe; colony lifting and hybridization was as described previously (36). The mouse TTRAP homologue was obtained by screening with BLAST (37) the EST data base for sequences homologous to human TTRAP. EST clone 1262914 (GenBank™ accession number AI465781) was requested from the IMAGE consortium (Cambridge, UK) and sequenced completely to obtain the mouse TTRAP cDNA sequence. The coiled coil prediction was obtained by running the program COILS (38).

Transient Transfections and Reporter Assays—293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g/liter) and 10% (v/v) fetal bovine serum. Transient transfection of plasmids for luciferase reporter assay or co-immunoprecipitation analysis was done with Fugene 6 (Roche Molecular Biochemicals), using 2 μl of Fugene per μg of plasmid DNA. For luciferase reporter assays, transfections were done in duplicate using 3×10^5 293T cells per well of a 24-well plate. Each well was transfected with 15 ng of reporter plasmid NFconluc, encoding the luciferase reporter gene driven by a minimal NF-κB-responsive promoter (gift of A. Israel, Institut Pasteur, Paris, France) or 50 ng of AP-1-luc (Stratagene, La Jolla, CA). To normalize the transfection efficiency, we co-transfected 75 ng of a *lacZ* reporter construct that contains the Rous sarcoma virus promoter inserted upstream of *Escherichia coli lacZ*. If the amount of TTRAP plasmid used in transfections was varied, we kept the total amount of DNA constant by adding a Myc-TTRAPmutant-pCS3 construct that does not produce TTRAP protein, because the TTRAP cDNA was cloned out-of-frame of the sequence encoding the N-terminal Myc tag. Cell extracts were prepared and assayed for luciferase activity and β-galactosidase activity according to the manufacturers' protocols (Promega (Madison, WI) and CLONTECH (Palo Alto,

CA), respectively). Data were normalized by calculating the ratio of luciferase and β-galactosidase activities. The average normalized luciferase activity is presented relative to the activity in nonstimulated samples as *x*-fold activation.

For co-immunoprecipitation, 1–2 × 10^6 293T cells were transfected with 2 μg of each expression vector. In the TRAF-TTRAP co-immunoprecipitation experiments, we noticed that overexpression of TRAFs 2, 3, 5, and 6 resulted in different synthesis levels of TTRAP from the co-transfected TTRAP-pcDNA3 construct. This was probably because of the fact that the cytomegalovirus promoter in the pcDNA3 vector (Invitrogen BV, Groningen, The Netherlands) is sensitive to the different levels of NF-κB induced by overexpressing these TRAFs. To circumvent this problem, we co-transfected 0.1 μg of hNIK-pcDNA3, which potently stimulates NF-κB.

Stimulation of cells with CD40L was done by overlaying transfected cells with mouse 3T6 fibroblasts stably transformed with an expression vector encoding hCD40L (39). As a negative control we used nontransfected 3T6 cells. Transiently transfected 293T cells at subconfluence were overlaid with approximately twice the number of 3T6 cells.

Co-immunoprecipitation and Western Blotting—Cells were harvested 24–48 h after transfection in 300 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, 50 mM NaF, 1 mM $Na_4P_2O_7$, 5 mM NA_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 3 μg aprotinin/ml). Cells were then lyzed by incubation for 20 min on ice or by passing five times through a 22-gauge needle. Cellular debris and nuclei were eliminated by centrifugation (Eppendorf 4517R, 14,000 rpm, 4 °C, 10 min). Five μg of antibody was added to the lysate and incubated for 3 h at 4 °C. Subsequently, 20 μl of a 50% slurry of protein G-Sepharose (Amersham Pharmacia Biotech, Gent, Belgium) was added to the samples, and the incubation was continued for 1 h. Next, the-Sepharose was washed four times in 750 μl of lysis buffer for 10 min at 4 °C. Finally, the beads were mixed with 20 μl of sample buffer, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis. To verify the expression levels of the different proteins, 0.1% of the cytoplasmic extract was analyzed on Western blot.

Proteins were separated on 12.5% Tris-Tricine gels and transferred onto polyvinylidene difluoride membrane (NEN Life Science Products) using a semi-dry blotting apparatus (Sigma). For Western analysis, the membrane was blocked in 3% skimmed milk in TBS-T (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20). After sequential incubation with primary and horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories, West Grove, PA) for 1 h at 24 °C, proteins were visualized with the ECL chemiluminescent detection system (NEN Life Science Products).

RESULTS

Cloning of a Novel CD40-binding Polypeptide—A two-hybrid screen in yeast was set up to identify novel CD40-interacting proteins. The cDNA encoding the cytoplasmic region of CD40 was cloned into the bait vector, which was transformed in yeast together with a HeLa cDNA library cloned in the prey vector. After screening approximately 2 × 10^6 transformants, eight different cDNAs were isolated from yeast colonies with a positive interaction phenotype. The corresponding eight polypeptides were tested for interaction with the cytoplasmic domain of other members of the TNF receptor family, *i.e.* human TNF-R75 and CD30. In addition, we also used as bait the C-terminal 192 amino acids of LMP1 from Epstein-Barr virus. Similar to CD30, CD40, and TNF-R75, LMP1 can signal through direct interaction with TRAFs (40, 41). One of the prey plasmids that was isolated in our screen contained a cDNA sequence encoding part of TRAF3 (amino acids 381–568, comprising part of the TRAF-N domain and the complete TRAF-C domain (9)). This hybrid prey protein associated with the cytoplasmic region of CD40, CD30, and LMP1, but not TNF-R75 (Table I), which is in accordance with published results for TRAF3 (10, 30, 41). Another positive prey, coded 4F2, bound to CD40, CD30, and TNF-R75 baits but not the LMP1 bait (Table I). The interaction phenotype of 4F2 with TNF-R75 was somewhat weaker than with CD40 and CD30. Also, the interaction phenotype of the latter two receptors with 4F2 was apparently not as strong as with the N-terminally truncated TRAF3 prey. The 1.8-kb-long partial cDNA for 4F2 encoded a novel polypeptide with no homology to TRAFs or other factors known to be involved in TNF receptor signaling.

TABLE I

Interaction test of TRAF3 (amino acids 381–566), 4F2, and TTRAP with the cytoplasmic domain of different receptors, using the yeast two-hybrid assay

The interaction phenotype was estimated by blue/white staining of yeast colonies. Staining was scored as blue, *i.e.* relatively strong and visible within 12 hours (++), strong and visible within 24 hours (+), or as white (−).

Bait	Prey		
	TRAF3 (381–568)	4F2	TTRAP
CD40	++	+	+
CD30	++	+	+
TNF-R75	−	+	+
LMP1	++	−	−

To obtain a full-length cDNA of this protein, we screened a HUVEC cDNA library using 4F2 cDNA as a probe and isolated a 2-kb-long cDNA. This yielded a complete open reading frame encoding a protein of 362 amino acids, that has been named TTRAP (TRAF and TNF Receptor-associated protein). Recently the complete genomic sequence of human TTRAP became available in the data base as a cosmid clone that maps to chromosome 6p22.1-22.3 (EBI accession number AL031775). The sequence of mouse TTRAP was obtained by sequencing EST clone 1262914, and the candidate *Caenorhabditis elegans* homologue of TTRAP was retrieved from the data base as putative protein predicted from the genomic sequence. Further comparison of TTRAP with the public data bases revealed that it is related to the C-terminal 380 amino acids of the yeast transcription factor CCR4 (42) and to a CCR4-like protein named nocturnin, which has been isolated from *Xenopus* (43) and recently also from human and mouse (44). CCR4 is distinct from TTRAP and nocturnin because it is approximately twice as big. The alignment of the TTRAP-related protein sequences shows that, although the overall amino similarities are rather low, there are stretches of identical amino acids scattered throughout the C-terminal 250 residues in the alignment (Fig. 1). The data in Table II furthermore indicate that nocturnin is more related to CCR4 than to TTRAP, whereas the *C. elegans* protein is more similar to TTRAP than to nocturnin. Taken together, our results indicate that TTRAP, nocturnin, and CCR4 belong to an emerging gene family. Neither nocturnin nor the C-terminal part of CCR4 has been characterized functionally.

The only known structural feature that could be deduced from the primary structure of TTRAP is a potential short coiled coil motif between amino acids 236 and 250 (Fig. 1), but this motif is poorly conserved in the *C. elegans* candidate TTRAP. Like 4F2, TTRAP also interacted with CD30, CD40, and TNF-R75 in the yeast two-hybrid assay (Table I). Interestingly, in the latter assay TTRAP also interacted with itself.

Distribution of Human and Mouse TTRAP mRNA in Adult Tissues and Embryos—We examined the expression of TTRAP mRNA using multiple tissue Northern blots, with 4F2 cDNA as a probe. In human tissues, a 2.2-kb transcript was observed in all tissues tested. In testis, an additional transcript of approximately 1.8 kb could be seen (Fig. 2*A*). The mouse TTRAP cDNA was used as a probe to screen murine blots. This revealed two transcripts of 3.4 and 2.2 kb, respectively (Fig. 2*B*), the larger of which is the more prominent. Like human TTRAP, mouse TTRAP mRNA was expressed in all of the adult tissues tested, but in heart and skeletal muscle the signal was very weak. In addition to adult expression, mouse TTRAP mRNAs are present in embryos throughout post-implantation, with somewhat a different ratio between the 2.2-and 3.4-kb bands

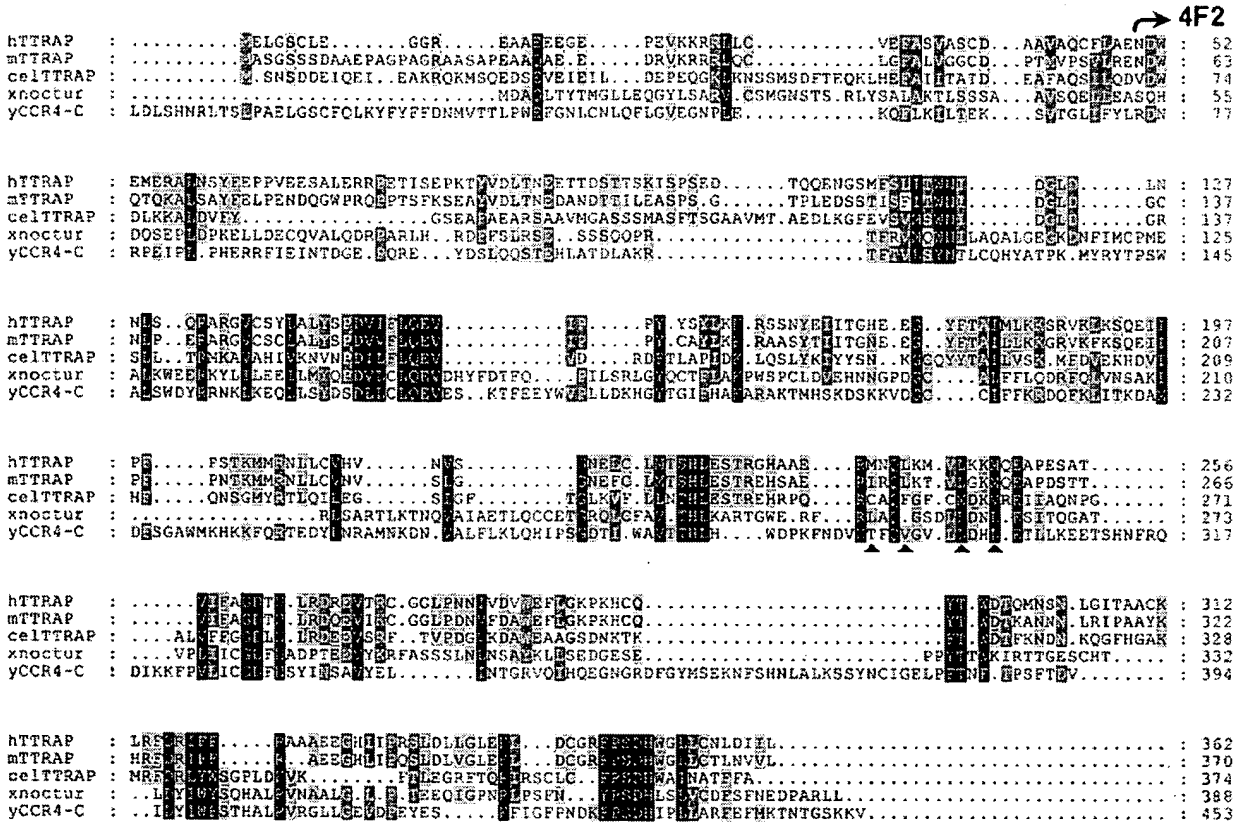

FIG. 1. **Alignment of the amino acid sequences of human TTRAP (*hTTRAP*), mouse TTRAP (*mTTRAP*), *C. elegans* TTRAP (*celTTRAP*), *Xenopus* nocturnin (*xnoctur*), and the C-terminal part of yeast CCR4 (*yCCR4-C*).** Residue 1 of yCCR4-C shown here corresponds to amino acid 385 of the full-length protein (57). The start of the partial human TTRAP polypeptide, 4F2, is indicated with a *thick arrow*. *Upright arrowheads* show the hydrophobic residues that could potentially be involved in the formation of a coiled coil region. *Boxed* amino acids are identical or physicochemically similar in 3 (*light gray*), 4 (*dark gray*), or all (*black*) of the aligned sequences.

TABLE II
Amino acid identity (percentage) for pair wise aligned protein sequences

The aligned sequences and their GenBank™/EBI accession numbers are: hTTRAP (human, AJ269473); mTTRAP (mouse, AJ251328); celTTRAP (*C. elegans*, CAA21707); hnocturnin (human, AAD56548.1); xnocturnin (*Xenopus laevis*, P79942); yCCR4-C (*S. cerevisiae*, amino acids 385–837, P31384).

	hTTRAP	mTTRAP	CelTTRAP	hnocturnin	xnocturnin
mTTRAP	69				
celTTRAP	35	38			
hnocturnin	32	32	30		
xnocturnin	32	29	33	75	
yCCR4-C	32	30	32	39	37

(Fig. 2*C*). *In situ* hybridization in sections of E12.5 mouse embryos showed that TTRAP mRNA was expressed ubiquitously (data not shown). At E15.5, widespread expression was weak, but stronger signals were observed in the kidneys, the small intestine, the seminiferous tubules of the testis, the lungs, the liver, brown fat, and the submandibular gland. The most striking expression was observed in the thymus lobes and in discrete regions of the brain (Fig. 2*D*).

TTRAP Interaction with CD40 Is Increased by Stimulation of Cells with CD40L—To confirm the interactions observed in yeast, we first tested co-immunoprecipitation of 4F2 with CD40 or TNF-R75 in cultured human cells. 293T cells were transfected with expression plasmids for FLAG-tagged 4F2 and either CD40, TNF-R75, or empty plasmid (as negative control). Extracts from transfected cells were then incubated with antibodies specific for CD40 or TNF-R75, followed by immunoprecipitation of these receptors. Subsequent Western blotting with anti-FLAG antibody revealed that 4F2 co-precipitated both with CD40 and TNF-R75 (Fig. 3*A*, *lanes 1* and *2* and *3* and *4*, respectively). We further examined whether cell stimulation with CD40L would affect the TTRAP-CD40 interaction. Expression vectors for CD40 and FLAG-TTRAP were co-transfected in 293T cells, and cells were stimulated with CD40L for a period of 2, 8, or 24 h. We repeatedly observed that treatment of the cells with CD40L for up to 24 h resulted in strongly increased TTRAP binding to CD40. In the experiment displayed in Fig. 3*B*, the amount of TTRAP co-immunoprecipitated with CD40 was densitometrically estimated to increase at least 10-fold after 24 h of stimulation. This could neither be explained by increasing levels of cellular production of TTRAP, which varied no more than 2-fold, nor was it because of increased precipitation of CD40 (Fig. 3*B*). In separate experiments (data not shown), this increase at 24 h even amounted to approximately 50-fold compared with the level of interaction at 8 h of stimulation.

The TTRAP Binding Site on CD40 Differs from That of TRAF3—The binding sites for TRAF2 and TRAF3 in the cytoplasmic domain of CD40 have been mapped to the PVQET motif (residues 250–254), whereas TRAF6 has been shown to bind to a distinct motif, KQEPQEINF (residues 230–238) (11, 45). In addition to binding TRAFs, CD40 interacts with JAK3 by a motif located N-terminal to the TRAF6 binding site (residues 222–229) (7). To map the region that binds TTRAP, a panel of CD40 tail mutants was tested as bait in a yeast two-hybrid assay. As a control prey, we used the partial TRAF3 that was picked in our original two-hybrid screening.

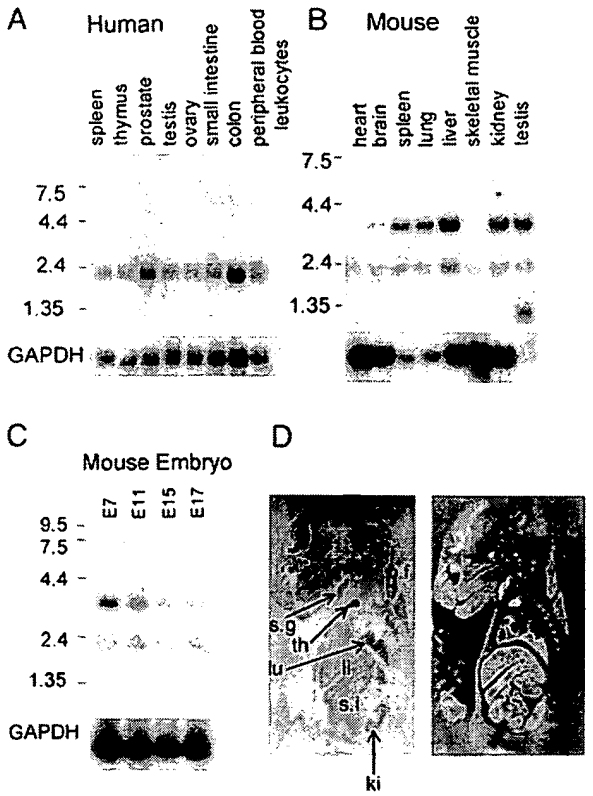

FIG. 2. **Analysis of TTRAP mRNA expression in adult human and murine tissues and in the murine embryo.** Northern blots from human (*A*) and mouse (*B, C*) tissues are shown. The blots were also hybridized with a rat probe for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *D, in situ* hybridization of *TTRAP* on a section of an E15.5 mouse embryo. Darkfield and brightfield images are shown in the *right* and *left panel*, respectively. Abbreviations: *b.f.*, brown fat; *ki*, kidney; *li*, liver; *lu*, lung; *th*, thymus; *s.g.*, submandibular gland, and *s.i.*, small intestine.

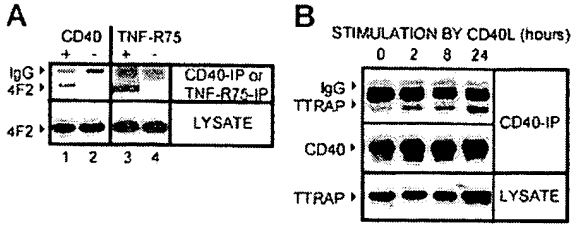

FIG. 3. **Co-immunoprecipitation of 4F2 and TTRAP with CD40 and TNF-R75.** *A*, FLAG-4F2-pcDNA3 (2 μg) was transiently transfected in 293T cells together with 2 μg of CD40-pcDNA3 (*lane 1*) or TNF-R75-pcDNA6 (*lane 3*) (both indicated as +) or empty vector (*lanes 2* and *4*; indicated as −). Immunoprecipitation was performed as described under "Experimental Procedures." *Top panels*, Western blot of 4F2-FLAG co-immunoprecipitated with CD40 (*lanes 1* and *2*) or TNF-R75 (*lanes 3* and *4*). The 5D12 or utr4 antibodies visible in the immunoprecipitates are marked IgG. *Lower panels*, synthesis level of 4F2-FLAG in lysates from transfected 293T cells. *B*, CD40-pcDNA3 (2 μg) was transiently transfected in 293T cells together with 2 μg of FLAG-TTRAP-pcDNA3. All cells were harvested at the same time point, *i.e.* 48 h after transfection, so the CD40L-expressing 3T6 fibroblasts were added to the 293T cells 24, 8, or 2 h prior to harvesting. The nonstimulated cells (0 h) were treated with 3T6 control cells for 2 h prior to harvesting. Co-immunoprecipitations were carried out as described under "Experimental Procedures." Co-precipitation of TTRAP-FLAG with CD40 is depicted in the *top panel*. *Middle panel*, immunoprecipitation of CD40. *Lower panel*, synthesis of TTRAP-FLAG in lysates from transfected 293T cells. In the *top* and *bottom panels*, proteins were detected with anti-FLAG antibody, and in the *middel panel*, anti-CD40 was used.

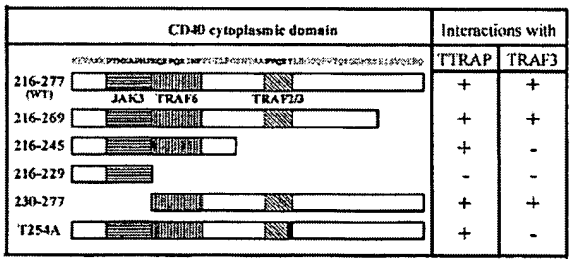

FIG. 4. **Interaction of TTRAP with CD40 requires the region between residues 230 and 245 in the receptor.** TTRAP binding to mutants of the cytoplasmic domain of CD40 was tested using the yeast two-hybrid mating assay as described under "Experimental Procedures." Different fragments of the human CD40 cytoplasmic domain, and the Thr-254 mutation to Ala (T254A), were used as bait constructs. Prey constructs were full-length TTRAP or N-truncated TRAF3 (amino acids 381–568). + indicates blue colonies after 24 h growth on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates; − indicates lack of color development after 48 h. Amino acids printed in *bold* represent known binding sites for JAK3, TRAF6, or TRAF2/3.

Shortening of the CD40 cytoplasmic tail from 62 residues (amino acids 216–277) to 30 amino acids (mutant 216–245) had no effect on TTRAP binding, whereas the same CD40 mutant no longer interacted with TRAF3 (Fig. 4). The latter result is in accordance with previous observations (11, 45). Further truncation of the cytoplasmic tail to 14 amino acids (mutant 216–229) abrogated TTRAP binding. Removing only these 14 amino acids of the cytoplasmic domain of CD40 (mutant 230–277) did not affect the binding of TTRAP. These results indicate that residues 230–245 of CD40 are required for its association with TTRAP. We also tested the Thr to Ala mutation at position 254, which is known to affect CD40 association with TRAFs 2, 3, and 5, as well as JAK3 (7, 45). TTRAP bound to this T254A mutant, whereas the partial TRAF3 did not (Fig. 4). In summary, these observations clearly show that the region of CD40 required for binding TTRAP differs from the one defined previously for TRAF2 and TRAF3, but may overlap with the interaction site mapped for TRAF6.

TTRAP Inhibits Activation of NF-κB Mediated by CD40 and TNF-R75—Overexpression of CD40 or TNF-R75 leads to activation of NF-κB (5, 10). To examine whether TTRAP may be involved in these signaling pathways, we investigated the effect of TTRAP overexpression on the activation of a reporter construct specific for NF-κB. Co-expression of TTRAP with these receptors inhibited NF-κB activation in a dose-dependent manner (Fig. 5). Typically, we observed that TTRAP overexpression decreased stimulation mediated by CD40 to a level of 20–30% of that in the absence of TTRAP. When using the CD40 T254A mutant, which signals through TRAF6 but not TRAF2 or TRAF5 (46), the inhibition by TTRAP was similar. In the case of TNF-R75, the effect of TTRAP ranged in the order of 40% residual NF-κB activity at the highest amount of TTRAP tested. To check whether overexpression of TTRAP influences the receptor expression levels, Western blots were carried out on the same lysates as used for the luciferase assays (Fig. 5, *insets*). Synthesis of CD40 or TNF-R75 did not decrease, indicating that TTRAP down-regulates their signal transduction normally leading to activation of NF-κB.

Interaction of TTRAP with TRAFs—CD40 has been shown to bind TRAFs 2, 3, 5, and 6 (9–12). The TRAFs are key players in the signal transduction cascade, and they interact not only with receptors but also with other effector proteins (8). We therefore investigated whether TRAFs could also interact with TTRAP. This was done by co-immunoprecipitation using protein extracts prepared from cells transfected with expression vectors for TTRAP-HA and FLAG-tagged human TRAFs 2, 3, 5, or 6. Immunoprecipitation from cell lysates was performed

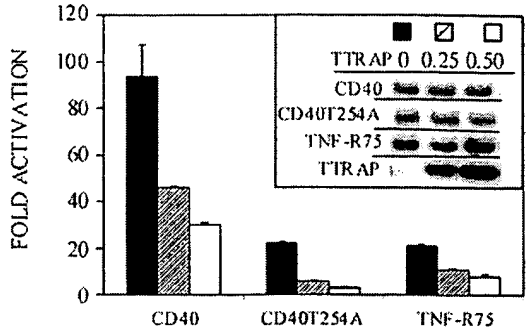

FIG. 5. **Inhibition of receptor-mediated activation of NF-κB by TTRAP.** 293T cells were transfected with an NF-κB-responsive luciferase reporter plasmid together with increasing amounts of TTRAP-pCS2 (0, 250, or 500 ng) and one of the following: 50 ng of CD40-pcDNA3, CD40(T254A)-pcDNA3, or TNF-R75-pcDNA6 and harvested after 48 h. Western blots in *insets* show CD40, TNF-R75, and TTRAP present in 5% of the cell lysates. Shown are the average values and S.D. of assays performed in duplicate, which are representative of at least three independent experiments.

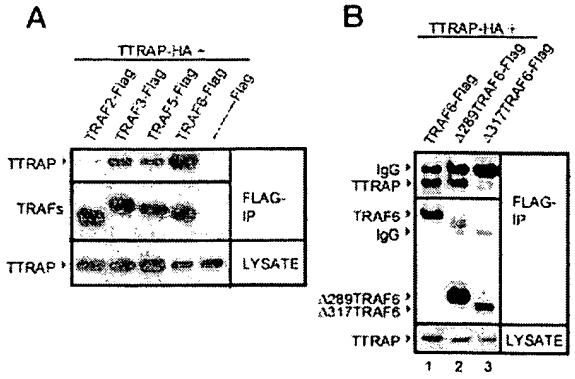

FIG. 6. **TTRAP co-immunoprecipitates with different TRAFs.** HA-TTRAP-pcDNA3 (2 μg) was transiently transfected in 293T cells together with 2 μg of FLAG-tagged wild-type or mutant TRAF constructs or empty FLAG vector. See "Results" for description of TRAF6 mutants. *A* and *B*, *top panels*: co-immunoprecipitation of TTRAP-HA with FLAG-tagged TRAF (mutants). The anti-FLAG antibody visible in the immunoprecipitates is marked IgG. *Middle panels*, immunoprecipitation of FLAG-tagged TRAFs. *Bottom panels*, synthesis of TTRAP-HA in lysates from transfected cells. Proteins were detected with anti-HA antibody (*top* and *bottom panels*) or anti-FLAG antibody (*middle panels*).

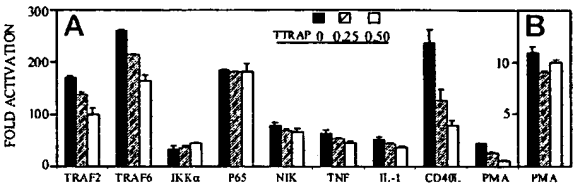

FIG. 7. **Effect of TTRAP overexpression on activation of NF-κB by different stimuli.** 293T cells were transfected with an NF-κB (*A*)-or AP-1 (*B*)-responsive luciferase construct, together with increasing amounts of TTRAP-pCS2 (0, 250, or 500 ng) and one of the following: 50 ng of FLAG-TRAF2-pRK5, 10 ng of FLAG-TRAF6-pRK5, 50 ng of HA-IKKα-pcDNA, 20 ng of Xpress-NIK-pcDNA3, 20 ng of P65/RelA-pRc, or 0.5 ng of CD40-pCDNA3. Alternatively, cells were stimulated with TNF-α (100 units/ml), IL-1β (100 units/ml), PMA (250 ng/ml), or CD40L for 24 h. In all cases, activation-fold was measured relative to cells not transfected with any stimulatory factor.

with anti-FLAG antibody. Subsequent Western blotting with anti-HA antibody revealed that TTRAP co-precipitated with all TRAFs tested, albeit with different efficiency (Fig. 6*A*). We repeatedly observed that TTRAP interacted more strongly with TRAF6 than with the other TRAFs, and this was not because of variation in efficiency of TRAF precipitation or TTRAP synthesis (Fig. 6*A*, *middle* and *bottom panel*, respectively). This suggests that TTRAP has a higher affinity for TRAF6 than for other TRAFs. In contrast to the ligand-dependent increase of TTRAP association with CD40 (Fig. 3*B*), its binding to TRAF6 was not affected by stimulation of the cells with CD40L for 2 or 24 h (data not shown). Because TTRAP interacted with TRAFs, we also tested whether the protein would associate with TRADD, an adaptor protein that links TRAFs to TNF-R55 (8). However, TTRAP could not be co-immunoprecipitated with transfected TRADD (data not shown).

To analyze which region in TRAF6 is required for binding TTRAP, we used TRAF6 deletion mutants. First, we tested the Δ289TRAF6 mutant that has no RING or zinc finger domains and therefore consists only of the TRAF domain (composed of TRAF-N and TRAF-C). TTRAP co-immunoprecipitated equally well with Δ289TRAF6 as with full-length TRAF6 (Fig. 6*B*, *upper panel*, *lanes 2* and *1*, respectively). By deleting 27 amino acids more, leaving only half of the TRAF-N domain and the complete TRAF-C domain (mutant Δ317TRAF6), the TRAF-TTRAP interaction was abrogated (Fig. 6*B*, *upper panel*, *lane 3*). Thus, in mammalian cells TTRAP can associate with TRAFs 2, 3, 5, and 6, with preference for the latter, and our data also show that the N-terminal half of the TRAF-N domain is required for TTRAP binding.

TTRAP Inhibits Activation of NF-κB Mediated by TRAF2, TRAF6, and PMA, but not by NIK, IKKα, or P65/RelA—It is known that overexpression of TRAFs 2, 5, and 6 leads to activation of NF-κB (10–12). To examine the effect of TTRAP co-expression on TRAF-mediated signaling, 293T cells were transfected with TRAF2 or TRAF6 and increasing amounts of TTRAP. TTRAP inhibited in a dose-dependent manner the TRAF-mediated activation of the NF-κB-dependent luciferase reporter (Fig. 7*A*), but the effect was not as profound as on receptor-mediated signaling (Fig. 5). Similar to TRAFs, overexpression of NIK, IKKα, and the NF-κB subunit P65/RelA induces transcription of the NF-κB reporter (18, 21). In contrast to our observations with CD40, TNF-R75, TRAF2, or TRAF6, TTRAP did not significantly affect NIK or IKKα-induced activation of NF-κB (Fig. 7*A*). Similarly, TTRAP overexpression had no effect on P65/RelA-mediated transactivation in the nucleus (Fig. 7*A*). These results suggest either that TTRAP exerts its inhibitory effect upstream of NIK or IKK-α mediated activation of NF-κB or that the pathway(s) involving these kinases are not affected by TTRAP.

TRAFs have also been implicated in signaling by TNF-R55 and the IL-1 receptor, therefore we tested the effect of TTRAP overexpression on NF-κB activation by these receptors by treating the cells with TNF-α and IL-1β. Because human embryonic kidney 293 cells do not express detectable levels of TNF-R75, TNF-α signals through TNF-R55 only (10). To compare the effect of TTRAP on stimulation by these ligands with its effect on CD40-mediated activation of NF-κB, cells were also stimulated with CD40L. Because 293T cells do not express the CD40 gene (45), we transfected relatively low quantities of receptor expression plasmid, so that ligand independent signaling contributed less than 5% to the CD40L-induced activation of NF-κB. TNF-α and IL-1β-induced activation of NF-κB was only modestly affected by TTRAP, whereas stimulation by CD40L was inhibited more profoundly (Fig. 7*A*) and to a similar extent as observed when overexpressing CD40 (Fig. 5). In addition to using specific ligands, we also treated the cells with the protein kinase C activator PMA. Luciferase reporter levels after stimulation with PMA were reduced to 20–30% at the highest TTRAP concentration tested (Fig. 7*A*). Because PMA also activates transcription of an AP-1-dependent reporter construct in 293T cells, it was analyzed whether TTRAP could also

inhibit AP-1 induced gene expression. Unlike its effect on PMA-mediated activation of NF-κB, TTRAP did not down-regulate AP-1-dependent gene activation by the phorbol ester (Fig. 7*B*), suggesting the inhibition by TTRAP may be restricted. Moreover, the fact that gene activation induced by P65/RelA or AP-1 was not sensitive to TTRAP indicates that the protein does not affect transcription in general. Taken together, these results demonstrate that TTRAP affects NF-κB induction in a dose- and stimulus-dependent fashion.

DISCUSSION

We report here on the isolation and characterization of the novel factor TTRAP that interacts with CD30, CD40, and TNF-R75, as well as with TRAFs 2, 3, 5, and 6. The binding properties of TTRAP resemble some of those described previously for the TRAFs and are indicative for a role of TTRAP in the signaling cascade induced by ligands of the TNF family. Structurally, TTRAP is unrelated to TRAFs or other intracellular proteins implicated in TNF signaling. The complete genome sequence for *Saccharomyces cerevisiae* and *C. elegans* is now available, and we found a candidate TTRAP homologue in the nematode but not in the yeast. Vertebrates express TTRAP and a related protein, named nocturnin, the precise function of which is unknown (43, 44). Another protein distantly related to TTRAP is the yeast factor CCR4, which is approximately twice as big as TTRAP and nocturnin (42). The domain homologous to the latter proteins spans the C-terminal 300 amino acids of CCR4, which has so far not been functionally characterized. Interestingly, this transcription cofactor has been shown by genetic analysis to be a potential downstream mediator of the protein kinase C1-MAPK signaling cascade in yeast (47). Therefore, our data suggest that TTRAP, nocturnin, and CCR4 are structurally related proteins with a potential role in signal transduction.

Judging from the quasi ubiquitous mRNA expression of TTRAP in adult mammals, its function may exceed an involvement in signaling by CD40 or TNF-R75. TRAFs 2, 3, and 6 mRNAs are also widely expressed in adult tissues (9, 11, 48), and mice deficient for each TRAF display severe problems during embryogenesis, clearly extending the importance of the latter signal transducers beyond the inflammatory response (8). The expression level and pattern of TTRAP mRNA during murine embryogenesis also suggests a role in development. For example, the strong mRNA signal in the embryonic thymus points toward a potential role for TTRAP in this organ.

Like the TRAFs, TTRAP binds to receptors of the TNF receptor family, *i.e.* CD30, CD40, and TNF-R75. However, unlike TRAF3, it does not associate with the Epstein-Barr virus protein LMP1. This demonstrates that TTRAP does not bind aspecifically to any receptor known to signal via TRAFs. To map the TTRAP binding site on CD40, we tested several deletion mutants of the cytoplasmic domain of the receptor and found the region between residues 230 and 245 to be required for TTRAP interaction. This stretch of 16 amino acids contains the TRAF6 binding motif, but is distinct from the site required for association of CD40 with TRAFs 2 and 3 (11, 45). This suggests that TTRAP and TRAF6 bind in close proximity or may even compete for the same region of CD40.

The interaction of CD40 with TTRAP apparently involves a mechanism that is different from what is known for the association of CD40 with TRAFs. Indeed, TRAFs 2 and 3 bind to the receptor within minutes after cell stimulation (49), whereas the recruitment of TTRAP to CD40 appears to be slower, with a continuous increase in time up to at least 24 h. Because in our experiments the TTRAP and CD40 protein levels do not change significantly upon CD40L stimulation, the increase in CD40-TTRAP interaction cannot result from an aspecific aggregation caused by the overexpression of TTRAP and/or CD40. This suggests that there is a CD40L-induced recruitment of TTRAP to CD40, which might involve activation or synthesis of one or more cofactors that assist or modulate the interaction. Alternatively, regulated proteolysis or decreased binding affinity of other receptor-interacting proteins may clear the way for TTRAP to form a complex with CD40.

TTRAP was isolated as a receptor-interacting protein, and therefore we were surprised to find that it also interacted with several TRAFs. We observed that TTRAP interacted more avidly with TRAF6 than with the other TRAFs. It is therefore possible that TTRAP is linked more specifically, but not exclusively, to TRAF6-mediated signaling events. Given the fact that TRAF6 is involved in signal transduction mediated by multiple receptors, *i.e.* CD40 (11), RANK (50, 51), Toll (14, 15), and the IL-1 receptor (16), the scope of TTRAP action could potentially be broader than shown by the results with CD40 and TNF-R75 reported here. Our observation that TTRAP partially inhibited NF-κB activation induced by IL-1β corroborates this possibility.

We have shown that TTRAP interacts with TRAF6 via the TRAF domain, which consists of a TRAF-N and-C subdomain. The former is a structurally conserved region that folds into an amphipathic helix that is required for the trimerization of TRAFs by forming a coiled coil with two other TRAF-N domains (52, 53). The TRAF-C domain is the most conserved region in this family of proteins and was shown to be involved in the direct interaction with different receptors, in trimerization of TRAFs, and in the association with other TRAF-binding proteins (8, 52, 53). Our experiments have shown that an intact TRAF-N domain is needed for binding TTRAP. Other proteins have previously been shown to require the TRAF-N domain for interaction with TRAFs, including the regulatory factors A20 (25), TRIP (28), and the cIAPs (54). Interestingly, TRAF2 can still bind to TNF-R75 when associated with TRIP or cIAPs, yielding a triple complex. Whether this is also the case with TTRAP remains to be investigated.

Recruitment of TTRAP to CD40 could either assist TRAFs and other proteins in triggering the signaling cascade or could counteract signal transduction to control cell stimulation with respect to duration and strength. The fact that overexpression of TTRAP inhibits activation of NF-κB mediated by CD40, TNF-R75, TRAF2, and TRAF6 supports the latter possibility. The inhibition by TTRAP may be the result of its interaction with receptors and/or TRAFs, whereby it (sterically) affects signal transduction. However, at this moment we cannot rule out the possibility that TTRAP acts (also) downstream of receptors and TRAFs. On the other hand, IKKα and P65/RelA-mediated activation of NF-κB was not down-regulated in our assays, suggesting that TTRAP inhibits processes upstream of IκB-phosphorylation by IKKα. NF-κB induction by NIK was also not significantly affected by TTRAP. In this respect, the action of TTRAP resembles that of another inhibitor of NF-κB activation, A20 (55). Our results imply either that NIK is downstream of TTRAP or that the pathway that is affected by TTRAP does not involve NIK. Evidence for the existence of such alternative but NIK-independent pathway stimulated by CD40 and involving TRAF6 was presented recently (46). It remains to be established whether TTRAP inhibits this particular signaling cascade.

Surprisingly, overexpression of TTRAP down-regulated NF-κB activation by the phorbol ester PMA. It is unclear how this activator of protein kinase C stimulates a plethora of cascades in the cell, but the fact that TTRAP did not prevent PMA-induced activation of the AP-1 reporter indicates that TTRAP affects processes downstream of the bifurcation of stim-

ulatory pathways for NF-κB and AP-1. This is again similar to what was shown for A20 (55). On the other hand, TTRAP and A20 differ by the fact that the effect of TTRAP depends on the receptor that is triggered, whereas this is not the case for A20. Indeed, the latter potently inhibits NF-κB activation by CD40, TNF, or IL-1 (55), whereas TTRAP had a more pronounced effect on stimulation by CD40L as compared with TNF or IL-1. Therefore, TTRAP does not seem to regulate all pathways involving TRAFs to the same extent. Signaling by TNF (through TNF-R55) is mediated by direct binding of TRAFs to the adaptor protein TRADD rather than to this 55 kDa receptor. TRAFs do, however, contact the receptors CD40 and TNF-R75 directly, and maybe overexpression of TTRAP affects this interaction more strongly than TRAF-TRADD interaction. This could be related to the fact that TTRAP does not bind to TRADD, whereas it does associate with CD40 and TNF-R75. Taken together, our current data demonstrate that overexpression of TTRAP results in a dose-dependent and stimulus-dependent inhibition of NF-κB.

Combining the observed inhibition of signal transduction with the increased recruitment of TTRAP to CD40 after ligand stimulation, we propose that TTRAP contributes to a negative feedback loop. Thus, by association with receptors and signaling factors, the protein would function to interfere progressively with gene activation, similar to inhibitory Smads in the transforming growth factor-β signaling pathway (56). However, at this point we cannot exclude that TTRAP could be a scaffold protein that tethers TRAFs, receptors, and maybe other signaling factors in the cell. In that case, TTRAP overexpression could also lead to decreased NF-κB activation, because instead of bringing the signal transducers together, it would separate them. Further research will be required to discriminate whether TTRAP functions as feedback inhibitor or scaffold protein in signaling by proteins of the TNF receptor and TRAF family.

Acknowledgments—We thank V. Dixit, D. Goeddel, J. Inoue, A. Israel, M. Karin, S. Plaisance, and M. Rowe for providing expression plasmids. We are grateful to R. Brent for sending us yeast strains, plasmids, and a cDNA library, which were used for two-hybrid screening. We also thank W. Buurman for providing antibodies and K. Thielemans for 3T6 (CD40L) cells.

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